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EXPRESSION ANALYSIS OF FKBP NUCLEIC ACIDS AND POLYPEPTIDES USEFUL IN THE DIAGNOSIS ~~AND TREATMENT~~ OF PROSTATE CANCER

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Fig. 2 is a flowchart demonstrating the procedure for RNA sample preparation, Affymetrix ~~Genechip~~ GENECHIP® (Human Expression Analysis Probe Array) hybridizations and analysis;

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The present invention is based, at least in part, on the identification of the genetic marker, FKBP54, which is differentially expressed in samples from androgen dependent prostate cancer cells. A panel of 6800 known genes was screened for expression androgen dependent prostate cancer cells (*see* Example 1). Those genes with statistically significant ($p<0.05$) differences between the diseased and normal tissues were identified. This differential expression was observed either as a decrease in expression, or an increase in expression. The expression of these selected genes in androgen dependent prostate cancer cells was assessed by ~~GeneChip~~ GENECHIP® (Human Expression Analysis Probe Array) analysis, as described in Example 1. FKBP54 was found to increase in expression in LNCaP prostate cancer cells. The growth of LNCaP cells and the production of PSA were responsive to a natural androgen receptor (AR) ligand, DHT, LNCaP and are suitable model for gene expression profiling. (*See e.g.*, Kokontis *et al.* (1994) *Cancer Res.* 54: 1566-1573; Schuurmans *et al.* (1991) *J. Steroid Biochem. Mol. Biol.* 40: 193-197; Swinnen *et al.* (1994) *Molec. Cell. Endocrinol.* 104: 153-162; Cleutjens *et al.*(1996) *J. Biol. Chem.* 271: 6379-6388; Henttu *et al.* (1992) *Endocrinology* 130: 766-772; Murtha *et al.* (1993) *Biochem.* 32: 6459-6464; Swinnen *et al.* (1996) Endocrinol. 137: 4468-4474).

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It will also be appreciated by one skilled in the art that the FKBP marker, e.g., FKBP54 of the invention may conveniently be provided on solid supports. For example, polynucleotides, such as mRNA, may be coupled to an array (*e.g.*, a ~~GeneChip~~ GENECHIP®, Human Expression Analysis Probe Array ~~array~~ for hybridization analysis), to a resin (*e.g.*, a resin which can be packed into a column for column chromatography), or a matrix (*e.g.*, a nitrocellulose matrix for northern blot analysis). The immobilization of molecules complementary to the marker(s), either covalently or noncovalently, permits a discrete analysis of the presence or activity of each marker in a sample. In an array, for example, polynucleotides complementary to the full length or a portion of the FKBP marker, e.g., FKBP54 marker may individually be attached to different, known locations on the array. The array may be hybridized with, for example, polynucleotides extracted from a skin cell sample from a subject. The hybridization of polynucleotides from the sample with the array at any location on the array can be detected, and thus the presence or quantity of the marker in the sample can be ascertained. In a preferred embodiment, a "~~GeneChip~~ GENECHIP® (Human Expression Analysis Probe Array)" ~~array~~ is employed (Affymetrix). Similarly, Western analyses may be performed on immobilized antibodies specific for the FKBP polypeptide (e.g., FKBP54) marker hybridized to a protein sample from a subject. In addition, quantitative PCR was used to confirm gene expression of the target marker. The transcription level of FKBP54 was found to be regulated by androgen, demonstrating a time dependent increase in transcription. Western blot analysis of the expressed FKBP54 protein further confirmed the time dependent increase in expression levels in the presence of androgen. The presence of FKBP54 in solid tumors was also demonstrated. Furthermore, transient

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and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package ~~(available at http://www.gcg.com)~~, using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package ~~(available at http://www.gcg.com)~~, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to marker protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. ~~See http://www.ncbi.nlm.nih.gov.~~

The invention also provides chimeric or fusion marker proteins. As used herein, a marker "chimeric protein" or "fusion protein" comprises a marker polypeptide operatively linked to a non-marker polypeptide. An "marker polypeptide" includes a polypeptide having an amino acid sequence encoded by the FKBP genes, e.g., FKBP54 gene, whereas a "non-

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(c) PSA ELISA

Quantification of PSA was performed using an ELISA. Briefly, a 96-well Nunc plate was coated with 100 µl of goat anti-PSA (1 µg/ml, Scripps laboratory, San Diego, CA) overnight at 4°C. The plate was washed with water three times and incubated with 100 µl of blocking buffer (PBS, 0.05% Tween 20, 1 µM EDTA, 0.25% BSA, and 0.05% NaN_3) for 1 h at room temperature. The plate was washed three times with water and incubated with 1:1 mixture of mouse anti-human PSA and Eu-labeled anti-mouse IgG (10 ng/antibody each/well for $1^1/2$ hs at RT). The plate was then washed four times with water. 100 µl of ~~Delfia~~ DELFIA® Enhancement Solution (to detect the binding of labeled ligands to receptors) (PerkinElmer Wallac Inc (Norton, OH) was added to the plate was read using a Victor reader (PerkinElmer Wallac Inc) according to the manufacturer's instruction.

(d) RNA extraction and preparation

Total RNA was isolated from LNCaP cells using the Qiagen ~~Rneasy~~ RNEASY® Midi Kit (a RNA extraction kit) following the manufacturer's recommendations. For polyA (+) selection, the Promega ~~PolyATract~~ POLYATRACT® (mRNA Isolation System with cDNA Synthesis Reagents) kit was used according to manufacturer's procedures. Briefly, LNCaP cells were collected by centrifugation and the RNA isolated using the buffers and recommended procedures from the Qiagen kit. Following RNA extraction, all samples were frozen at –80°C.

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Affymetrix ~~Genechip™~~ GENECHIP® (Human Expression Analysis Probe Array) technology was used to monitor the expression of about 6000 full-length human genes in response to a natural androgen DHT in LNCaP cells. Fig. 2 illustrates the general scheme used for sample preparation, hybridization, and analysis. Hybridization cocktail was made using 10 μg of fragmented cRNA, 2X MES buffer with BSA, herring sperm DNA, control prokaryotic transcripts for internal control, and biotinylated control oligo 948 (for chip quality control). DEPC-water was added to bring the

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volume to 200 µl. Prior to hybridization, the hybridization cocktails were heated to 99°C for 10 minutes, and then 37°C for an additional 10 minutes before loading into Hu6800FL arrays (Affymetrix ~~GeneChips™~~ GENECHIP® (Human Expression Analysis Probe Array)). The Hu6800Fl array is comprised of 6800 known full-length genes, about 250,000 25-mer oligonucleotide probes with 20 probe pairs per gene. Array hybridization proceeded overnight at 45°C with 50 rpm. Following hybridization, the arrays were washed and stained using the manufacturer's recommendations and procedures. (Affymetrix Expression Analysis Technical Manual). Non-stringent wash buffer (20X SSPE, 1.0ml of 10% Tween 20, and water) at 25°C, and stringent wash buffer (20X SSPE, 5M NaCl, 10% Tween 20, and water) at 50°C were used for the wash steps. The arrays were then stained with strepavidin-conjugated phycoerythrin (SAPE, Molecular Probes), followed by biotinylated anti-strepavidin and a second round of SAPE for signal amplification at 25 °C. Each stain step was done for 10 minutes. All arrays were then scanned using the HP Genearray Scanner and the resulting fluorescence emmisions were collected and quantified using Affymetrix Genechip software. Within the software, the signal intensities for all the probes on each array were calculated from the scanned image, and the appropriate probe array algorithm was applied to determine the expression levels (average difference) for each gene. Average differences for all genes were converted into mRNA frequency estimates (in molecules per million) based on the standard spike-in control transcripts.

(f) Data Filtering and Statistics

Initial data was reduced by filtering for all genes called "present" by ~~GeneChip™~~ GENECHIP® (Human Expression Analysis Probe Array). A two-way ANOVA was then performed on the replicate data for each of these genes in the statistical computing package S-plus. The potential effects of two experimental factors (treatment and time) and the interaction of both factors on the expression level were evaluated in the analysis of variance model, and the p-values for the main effects ($P_{treatment}$, P_{time}) and for the interaction($P_{interact}$) were obtained. Only those genes that were statistically significant (p-value $<= 0.05$) for the treatment factor and/or the interaction were considered for the time being. First, the average was taken for baseline and experimental replicate mRNA

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frequencies of the 705 genes that passed this p-value criterion. Average frequencies obtained for each gene were then standardized across all samples to have a mean of zero and a standard deviation of one. A modified version of the original self-organizing map (SOM) algorithm developed by Kohonen *et al* (Self-Organizing Maps, Second Extended Edition edition, Vol. 30. New York, 1997), created using the MATLAB toolbox, was then applied to the standardized expression values to generate a 6 by 6 matrix of 36 clusters (Tamayo *et al.* (1999) *Proc. Natl. Acad. Sci. USA.* 96: 2907-2912). Several public databases such as Genecards and Swiss-Prot were used for gene annotation (*See e.g.,* Rebhan *et al.* GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science, Bioinformatics Unit and Genome Center (Rehovot, Israel), 1997~~. World Wide Web URL: http://bioinfo.weizmann.ac.il/cards~~, and Appel *et al.* (1994).A new generation of information retrieval tools for biologists: ~~the example of the ExPASy WWW server.~~*Trends Biochem. Sci.* 19:258-260 ~~World Wide Web URL: http://www.expasy.ch/sprot/~~).

(g) Quantitative ~~Taqman~~ TAQMAN® RT-PCR

The same total RNA samples used for the ~~GeneChip~~ GENECHIP® (Human Expression Analysis Probe Array) experiments were analyzed using a ~~*Taqman*®~~ TAQMAN® EZ RT-PCR kit (a one-step RT-PCR kit). (PE Applied Biosystems) to confirm gene expression changes. Total RNA samples were diluted to a concentration of 50ng/ul and a total of 50 ng was used for each reaction. Primers and florescence probes for PSA and FKBP54 were designed using the Primer Express software and were chosen based upon the manufacturer's recommendations for primer selection. The primers used were of 100uM concentration and were as follows: (a) PSA-F (forward primer) CGTGGCCAACCCCTGA (SEQ ID NO: 1), PSA-R (reverse primer) CTTGGCCTGGTCATTTCCAA (SEQ ID NO: 2), and PSA-P (probe) CACCCCTATCAACCCCCTATTGTAGTAAACTTGGA (SEQ ID NO: 3).
(b) FKBP54-F (forward primer) CTGTGACAAGGCCCTTGGA (SEQ ID NO: 4), FKBP54-R (reverse primer) CTGGGCTTCACCCCTCCTA (SEQ ID NO: 5), and FKBP54-P (probe) ACAAGCCTTTCTCATTGGCACTGTCCA (SEQ ID NO: 6).

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Samples were prepared using a reagent mix of manufacturer supplied RT-PCR components [(5X ~~TaqMan~~ TAQMAN® (a one-step RT-PCR kit) EZ Buffer, manganese acetate (25 mM), dATP (10mM), dCTP (10mM), dGTP (10mM) and dUTP (20mM), rT*th* DNA polymerase (2.5U/μl), AmpErase UNG (1 U/μl), primers (final concentration 1μM) and RNA (50ng)], following manufacturer's recommendations. In addition, GAPDH control samples for standard curve generation and subsequent quantitation of sample RNA was prepared. Primers and probe for GAPDH were included in the kit (GAPDH forward and reverse primers 10μM, GAPDH probe 5 μM). β-actin was also used for standard curve generation, and dilutions were made for both genes that ranged from 5 X 10^6 copies to 5 X 10^1 copies. The assay was performed on a Perkin-Elmer/Applied Biosystems 7700 Prism, and the PCR cycling parameters were chosen based on the manufacturer's recommendations. RNA of samples were normalized to GAPDH and β-actin and was quantified.

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(k) Luciferase Assay

Luciferase activity was determined using Promega's ~~Steady-Glo~~ STEADY GLO™ Luciferase Assay System (a luciferase assay system designed for the purpose of providing long-lived luminescence when added to cultured cells). Briefly, after 24 hours of treatment, cells were harvested by scraping in 1ml of PBS. 5 µg protein from each sample in a total of 100 µl PBS was ~~mixmed~~ mixed with 100 µl of ~~Stable-Glo~~ STEADY GLO™ reagent (Promega), and luminescence was determined in a luminometer (Wallac, 1450 MicroBeth Counter) after 5 min.

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Genechip Hybridization and Analysis

Affymetrix ~~Genechip™~~ GENECHIP® (Human Expression Analysis Probe Array) technology was used to monitor the expression of about 6000 full-length human genes in response to a natural androgen DHT in LNCaP cells. Fig. 2 illustrates the general scheme used for sample preparation, hybridization, and analysis and the details of hybridization are described in section (e). To obtain reliable data, total RNA was prepared in duplicate from LNCaP cells treated or not with DHT for 0, 2, 4, 6, 12, 24, 48, and 72 hs as described in section (d). CRNAs were prepared and hybridized also in duplicate to Affymetrix chips. Therefore a set of biological replicates for a total of 30 samples were generated for each experiment to ensure reproducibility. Only those genes that were called "present" in either the baseline or the experiment in at least one time point and in either replicate passed the initial data reduction filter. Out of about 6000 genes represented on the chip, 4491 passed this initial filter (75%).

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Quantitative RT-PCR analysis of RNA samples

Quantitative RT-PCR was also used to confirm the gene expression changes from the ~~GeneChip~~ GENECHIP® (Human Expression Analysis Probe Array) analysis as described in section (g). The results for qualitative RT-PCR are shown in Fig. 4 A and B, demonstrating the increase in RNA levels for PSA and FKBP54.